Loss of Freezing Tolerance Associated with Decrease in Sugar Concentrations by Short-term Deacclimation in Cabbage Seedlings

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Summary
Cabbage seedlings cold-acclimated for 8 days at 5°C were transferred to 15, 20 or 25°C in the dark or light. The acquired freezing tolerance was reversed during deacclimation at all temperatures; the higher the deacclimation temperature, the more accelerated was the loss of freezing tolerance. Concentrations of soluble sugars, particularly sucrose in the leaves, rapidly decreased within deacclimation for 1 to 3 hr at 20°C in the dark. This reduction was accompanied with a decrease in the freezing tolerance of leaves. Activities of soluble acid invertase in leaves were not affected by short-term deacclimation.

Key Words: Brassica oleracea L., cold acclimation, deacclimation, freezing tolerance, sugar concentration.

Introduction
There are seasonal changes in the freezing tolerance of plants (Alberdi and Corcuera, 1991; Jung and Smith, 1961; Perras and Sarhan, 1984). In cabbage, winter cold injures plants when the temperature exceeds the its freezing tolerance. However, it is a serious problem in Japan when an early spring frost damages cabbages (Kikkawa et al., 1979). Spring frost injury of cabbage often occurs when deacclimated plants are exposed to low temperature. Therefore, it is important for vegetables to remain cold-acclimated to forestall deacclimation.

Arabidopsis becomes cold-hardy to a significant degree after only 12 hr at low temperature (Gilmour et al., 1988). In cabbage seedlings, Sasaki et al. (1996) found that freezing tolerance was acquired within 1 week by exposure to non-freezing low temperature. On the other hand, Mohapatra et al. (1987) reported that alfalfa seedlings, acclimated to cold for 17 days, completely lost their freezing resistance by 2 days of deacclimation. Sasaki et al. (1996) reported that the acquired freezing tolerance is reduced within 1 day by returning plants to moderate temperature.

Cold acclimation is accompanied by biochemical changes such as accumulations of carbohydrates, amino acids, glycinin, and polyamines (Jung and Smith, 1961; Perras and Sarhan, 1984; Alberdi and Corcuera, 1991). Sucrose is the most commonly accumulated free sugar in response to low temperature (Guy et al., 1992) but it is rapidly metabolized during deacclimation of a few days, accompanied by changes in activities of enzymes (Tognetti et al., 1989). However, environmental conditions that induce deacclimation and sugar metabolism during several hours of deacclimation have not yet been studied sufficiently although acclimation is important for prevention injury to cabbage by an early spring frost. Griffith and McIntyre (1993) reported that frost tolerance of winter wheat during cold acclimation is dependent upon irradiance, which affects the amount of photoassimilates available, but they did not investigate necessity of light during deacclimation.

The objective of the present study was to investigate effects of temperature and light on freezing tolerance and sugar concentration in cabbage seedlings during short-term deacclimation.

Materials and Methods

Plant material and treatments
Seeds of cabbage (Brassica oleracea L. cv. Banchurisou) were sown in plastic pots filled with a soil mixture (Pretty Soil Gold N-140, Otsukasangyo, Nagano, Japan). Plants were grown in a growth chamber at 20/15°C (day/night) under a 12-hr photoperiod, supplied by metal halide lamps (MLBC400C-U, Mitsubishi Electric OSRAM, Yokohama, Japan); the photosynthetic photon flux density (PPFD) was 230 ± 10 μmol·m⁻²·sec⁻¹. For acclimation, seedlings were grown for 3
weeks at 5 °C as above for 7, 8, or 10 days.

For deacclimation, the cold-acclimated seedlings were transferred after 7 days to 20 °C under 230 ± 10 μmol·m⁻²·sec⁻¹ PPFD or dark for 1 day.

Cold-acclimated seedlings were transferred to 15, 20 or 25 °C after 8 days and kept in the dark to deacclimatize for 0 to 12 hr, after which their freezing tolerance was measured.

To determine carbohydrate concentration, seedlings cold-acclimated for 10 days were deacclimated at 20 °C for 0 to 6 hr. After the deacclimation treatments, the sugar concentrations, invertase activity, and freezing tolerance of the cabbage leaves were determined.

**Freezing tolerance test**

Freezing tolerance was evaluated according to Sasaki et al. (1998) by excising two leaf discs (10 mm in diameter) from the second leaves from each of 4 seedlings. Two leaf discs were placed in a test tube and transferred to a chamber where the temperature was lowered from 15 to −8 °C at a rate of 0.25 °C·min⁻¹. The leaf discs were then sprayed with deionized water to initiate extracellular freezing. The test tubes were maintained at −4, −6 or −8 °C for 30 min and then allowed to thaw at room temperature for about 1 hr. Deionized water (15 ml) was added and the tubes were stored overnight at room temperature. The conductivity of the solution was measured before and after the samples were boiled for 20 min. The degree of electrolyte leakage was calculated as the conductivity of the solution before heating as a percentage of that after heating.

**Determination of sugar contents and invertase activity**

The second leaves were cut from the two plants and their midribs removed. The remainder of the leaf was extracted with 80% ethanol and soluble sugar contents measured as previously described (Sasaki et al., 1998) using HPLC.

Leaf tissue was cut into small discs, which were frozen in liquid N₂ and stored at −80 °C. All steps were performed at 4 °C unless otherwise stated. Frozen leaf discs were homogenized in 50 mM HEPES-KOH buffer (pH 8.0), containing 5 mM β-mercaptoethanol, 2 mM EDTA, and 2% polyvinylpolypyrrolidone. The homogenates were centrifuged at 15,000 x g for 10 min and supernatants were immediately applied to NAP-10 columns, equilibrated with 20 mM HEPES-KOH buffer (pH 8.0), 6 mM β-mercaptoethanol, and 2 mM EDTA. Invertase activity was assayed by determining the amount of glucose liberated from sucrose by the glucose oxidase method. Enzyme preparation was added to a reaction medium composed of 60 mM acetate acid buffer (pH 5.0) and 100 mM sucrose, and incubated at 30 °C for 45 min. The reaction was stopped by boiling the reaction mixture with 0.2 volume of 300 mM Tris-HCl buffer (pH 8.0).

**Results**

When leaves of cabbage which had been exposed to low temperature were subjected to freezing tolerance at −4 °C, the values of electrolyte leakage before and after cold acclimation were 85 and 41%, respectively (Table 1). The result indicates that the cabbage seedling had acquired freezing tolerance. The electrolyte leakage from the leaves of the plants exposed to 20 °C for 1 day increased regardless of the light condition. This indicates that the acquired freezing tolerance is lost and deacclimation is not light-dependent.

Experiments, seeking the relationship between freezing tolerance of cabbage seedlings and temperature for deacclimation, revealed that freezing tolerance induced by cold acclimation at 5 °C for 8 days was reversible by exposure to 15, 20 or 25 °C (Fig. 1). The value of electrolyte leakage after freezing at −6 °C increased as the period of deacclimation treatment was longer (Fig. 1B). The higher the temperature for deacclimation, the greater was the electrolyte leakage, indicating that acquired freezing tolerance is reduced by a short period of deacclimation and that the degree of deacclimation is temperature-dependent.

Soluble sugar concentrations of the seedlings were increased by a 10-day cold acclimation. Sucrose, glucose and fructose increased from 0.39 to 2.3, from 1.2 to 6.8 and from 1.4 to 6.9 mg·g⁻¹FW, respectively. Glucose and fructose concentrations decreased continuously during deacclimation; sucrose concentration decreased more rapidly than the hexoses at the onset of deacclimation (Fig. 2A). However, invertase activity in leaves did not change during short-term deacclimation (Fig. 2B). Deacclimation at 20 °C for 3 hr increased the electrolyte leakage after freezing at −6 and −8 °C indi-

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<th>Table 1. Freezing tolerance of leaves of cabbage seedlings exposed to 20 °C for 24 hr following cold acclimation at 5 °C for 7 days. Freezing tolerance is expressed in terms of percentage electrolyte leakage from leaves after freezing test at −4 °C, −6 °C and −8 °C.</th>
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Values are means ± standard errors (4 replications). Means indicated by different letters within a column are significantly different at 0.05 or 0.01 level by LSD.
Fig. 1. Effect of short-term deacclimation on freezing tolerance of cabbage leaves. Freezing tolerance test at -4°C (A), -6°C (B), and -8°C (C). Vertical bars indicate SE (n=4).

Fig. 2. Effect of short-term deacclimation at 20°C on soluble sugar concentrations (A) and invertase activity (B) of cabbage leaves. Vertical bars indicate SE (A: n=3, B: n=6).

Fig. 3. Changes in freezing tolerance of cabbage leaves during deacclimation. Vertical bars represent SE (n=4).

Discussion

Although low temperature induced little or no hardening of cabbage in the absence of light (Cox and Levitt, 1976), freezing tolerance of cold-acclimated plants was reduced by exposure to 20°C in light and dark (Table 1) which suggest that the light is not required for deacclimation of cabbage seedlings. Nonetheless, the values of electrolyte leakage of plants deacclimated under dark tended to be higher than those under light (Table 1). So that the loss of freezing tolerance by deacclimation might be affected by light. The degree of freezing tolerance depends on the sugar concentration of plants (Gusta et al., 1996; Sasaki et al., 1996). Since plants can not fix carbon by photosynthesis in the dark, sugar concentration in darkened leaves may decrease faster than that under light, resulting in a rapid loss of freezing
tolerance.

In this study, concentrations of sugar, especially sucrose, tended to decrease during the 1 hr deacclimation (Fig. 2A). Metabolism of sugars during acclimation has been studied in some plants (Carderón and Pontis, 1985; Salerno and Pontis, 1989; Tognetti et al., 1989; Guy et al., 1992). However, their results are not always consistent. Invertase activity in cabbage seedlings did not change during a short period of deacclimation (Fig. 2B), although sucrose concentration decreased (Fig. 2A). Guy et al. (1992) reported that decreased invertase activity in spinach exposed to 5 °C tended to increase when kept at 25 °C for 7 days. Why invertase activity differs among researchers is not clear. Glucose and fructose also decreased during deacclimation (Fig. 2A); consistent with findings with various species (Steponkus and Lanphear, 1968; Guy et al., 1992; Tronsmo et al., 1993). We attribute this decrease to accelerated deacclimation or the restart of growth so that hexoses were rapidly consumed directly or indirectly via the glycolytic pathway for energy production.

Sasaki et al. (1996) reported that the period required for deacclimation is much shorter than that for acclimation in cabbage. In this study, freezing tolerance started to decrease by exposure to 20 °C within 3 hr. The rate of decrease depended on the temperature of deacclimation. These results suggest that a short temporary rise of temperature on a sunny day might reduce the freezing tolerance of cabbage in the field and cause the cold injury. In Japan, frost injury of cabbage often happens in spring rather than in winter (Kikkawa et al., 1979). Hence, analyses of deacclimation and changes in freezing tolerance of cabbage in the field after a temporary rise in temperature are important.

In conclusion, freezing tolerance acquired by cold acclimation was reduced by exposure to deacclimation of 3 to 6 hr without light. Loss of freezing tolerance was accelerated more when the temperature for deacclimation was higher. Sugar concentrations respond to changes in freezing tolerance in seedlings during deacclimation.

**Literature Cited**


キャベツ幼植物の短時間脱順化による糖濃度の低下を伴った耐凍性の消失

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摘 要

5℃処理8日間で低温順化したキャベツ幼植物を15、20および25℃の暗条件、しくは明条件に移した。獲得されていた耐凍性は、すべての昇温処理で消失した。脱順化処理の温度が高いほど、耐凍性の消失は速やかであった。可溶性糖、特にスクロースは、短時間脱順化処理（20℃暗条件、1-3時間）で急激にある程度まで減少し、こうした糖質の減少は、葉の耐凍性の低下と同じ挙動であった。葉の可溶性酸性インペルターゼ活性は、短時間脱順化処理によって影響を受けなかった。

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